Segmental expression of Hoxa-2 in the hindbrain is directly regulated by Krox-20

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The hindbrain is a segmented structure divided into repeating metameric units termed rhombomeres (r). The Hox family, vertebrate homologs of the Drosophila HOM-C homeotic selector genes, are expressed in rhombomere-restricted patterns and are believed to participate in regulating segmental identities. Krox-20, a zinc finger gene, has a highly conserved pattern of expression in r3 and r5 and is functionally required for their maintenance in mouse embryos. Krox-20 has been shown to directly regulate the Hoxb-2 gene and we wanted to determine if it was involved in regulating multiple Hox genes as a part of its functional role. Hoxa-2 is the only known paralog of Hoxb-2, and we examined the patterns of expression of the mouse Hoxa-2 gene with particular focus on r3 and r5 in wild type and Krox-20-/- mutant embryos. There was a clear loss of expression in r3, which indicated that Hoxa-2 was downstream of Krox-20. Using transgenic analysis with E. coli lacZ reporter genes we have identified and mapped an r3/r5 enhancer in the 5' flanking region of the Hoxa-2 gene. Deletion analysis narrowed this region to an 809 bp BgIII fragment, and in vitro binding and competition assays with bacterially expressed Krox-20 protein identified two sites within the enhancer. Mutation of these Krox-20 sites in the regulatory region specifically abolished r3/r5 activity, but did not affect neural crest and mesodermal components. This indicated that the two Krox-20 sites are required in vivo for enhancer function. Furthermore, ectopic expression of Krox-20 in r4 was able to transactivate the Hoxa-2/lacZ reporter in this rhombomere. Together our findings suggest that Krox-20 directly participates in the transcriptional regulation of Hoxa-2 during hindbrain segmentation, and is responsible for the upregulation of the r3 and r5 domains of expression of both vertebrate group 2 Hox paralogs. Therefore, the segmental phenotypes in the Krox-20 mutants are likely to reflect the role of Krox-20 in directly regulating multiple Hox genes.

Key words: Krox-20, Hoxa-2, hindbrain segmentation, rhombomeres, transcriptional regulation, transgenic mice, enhancers

INTRODUCTION

Segmentation is an important mechanism in regionalisation of the hindbrain which has been highly conserved during vertebrate evolution (Lumsden, 1990; Wilkinson, 1993; Keynes and Krumlauf, 1994). In early vertebrate embryos a similar number of periodic swellings transiently appear in the developing hindbrain where they are termed rhombomeres (r). Cellular analysis in chicken embryos has demonstrated that rhombomeres are lineage-restricted compartments (Fraser et al., 1990; Birgbauer and Fraser, 1994), with reduced mixing and cell-cell communication between adjacent segments (Guthrie and Lumsden, 1991; Martinez et al., 1992; Guthrie et al., 1993). There is a tight correlation between specific rhombomeric segments and the organisation of branchiomotor nerves, sensory ganglia, neuronal development, branchial arches and generation/migration of cranial neural crest (Lumsden and Keynes, 1989; Lumsden et al., 1991; Serbedzija et al., 1992; Sechrist et al., 1993; Birgbauer et al., 1995). On the basis of axonal organisation of the branchiomotor and sensory nerves (Lumsden and Keynes, 1989) and that alternate rhombomeres have similar properties with respect to cell mixing and neural crest generation and migration (Guthrie and Lumsden, 1991; Graham et al., 1993, 1994; Guthrie et al., 1993; Sechrist et al., 1993), there appears to be an underlying two-segment periodicity to the general organisation of the hindbrain. Together this cellular data strongly argues that rhombomeric segments are fundamental units involved in generating regional diversity in the CNS and in head morphogenesis.
Underlying this morphological organisation, at the molecular level expression studies have revealed that transcription factors, growth factors and receptor tyrosine kinases display rhombomere-restricted patterns of expression (Wilkinson et al., 1988, 1989a, b; Gilardi-Hebenstreit et al., 1992; Becker et al., 1994; reviewed by Wilkinson, 1993). In vertebrates, many members of the Hox gene family have anterior limits of expression in the hindbrain which map precisely to rhombomere boundaries, and in addition there are high levels of expression in specific rhombomeres (Murphy et al., 1989; Wilkinson et al., 1989b; Sundin and Eichele, 1990; Hunt et al., 1991; Prince and Lumsden, 1994; reviewed by McGinnis and Krumlauf, 1992; Keynes and Krumlauf, 1994). Furthermore these restricted patterns of Hox expression arise before the morphological appearance of rhombomeres, suggesting that Hox genes play a role in regulating rhombomeric processes. Loss-of-function mutations in the mouse Hox-1 gene, generated by targeted disruption, severely affect hindbrain patterning, and in particular, formation of r5 (Carpenter et al., 1993; Dolle et al., 1993; Mark et al., 1993). However, no overt rhombomeric phenotypes have been observed in other targeted Hox mutations (reviewed by Krumlauf, 1994), which could be due to functional compensation or synergistic interactions between different Hox genes expressed in the same region (Condie and Capecchi, 1994). In gain-of-function experiments ectopic expression of the Hoxa-1 gene results in the transformation of r2 to an r4 identity (Zhang et al., 1994), demonstrating that in a manner analogous to their Drosophila HOM-C counterparts, the Hox genes can regulate segmental identity.

Very little is known about how the rhombo meric-restricted patterns of Hox expression are established in the hindbrain. Krox-20 encodes a zinc finger transcription factor (Chavrier et al., 1988, 1990) and is expressed in the presumptive r3 and r5 domains of many vertebrate embryos (Wilkinson et al., 1989a; Nieto et al., 1991; Bradley et al., 1992; Oxtoby and Jowett, 1993), and loss-of-function mutations in the mouse Krox-20 gene severely affect these rhombomeres (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993). In the case where the Krox-20 gene has been disrupted by insertion of the E. coli lacZ gene, it is possible to follow Krox-20 expressing cells in the mutants. In these embryos we have observed that r3 and r5 are initially formed, but fail to develop properly and are rapidly eliminated (Schneider-Maunoury et al., 1993). Hence Krox-20 is required for the maintenance of r3 and r5. Krox-20 has been shown to be directly involved in the regulation of the Hoxb-2 gene in r3 and r5 (Sham et al., 1993), which links the Krox-20 mutant phenotype with the regulation of Hox genes. However, it is unclear whether the segmental abnormalities in the mutant mice arise from the influences on Hoxb-2 alone or involve other members of the Hox family.

The four vertebrate Hox clusters arose by duplication and divergence from a common ancestor, and as a consequence there are highly related genes in each of the complexes which represent paralogous groups (McGinnis and Krumlauf, 1992). Not only are paralogous genes similar in structure, but in the hindbrain they have similar rhombomere-restricted patterns of expression (Hunt et al., 1991; Keynes and Krumlauf, 1994), suggesting a real potential for functional compensation or redundancy. The only paralog of Hoxb-2 is Hoxa-2, and these genes have different anterior boundaries of expression in the neural tube in both chicken and mouse embryos where Hoxa-2 is present in r2 (Krumlauf, 1993; Prince and Lumsden, 1994; Frasch et al., 1995). Despite these differences it has been shown in the mouse that Hoxa-2 is also expressed at higher levels in r3 and r5 (Hunt et al., 1991; Krumlauf, 1993). Therefore, Krox-20 might also be involved in regulating this gene, although loss-of-function mutations in the Hoxa-2 gene do not display abnormalities in r3 and r5 (Gendron-Maguire et al., 1993; Riijli et al., 1993).

In this study we have examined the expression of Hoxa-2 in homozygous Krox-20 mutants and found that it is altered, suggesting that it is downstream of Krox-20. Furthermore, we have used a combination of transgenic and in vitro biochemical analysis to identify an r3/r5 enhancer in the Hoxa-2 locus, and show that this enhancer has Krox-20 binding sites necessary for in vivo activity. The results demonstrate that Hoxa-2 is also a direct target of Krox-20 during hindbrain segmentation, indicating that Krox-20 is involved in the control of multiple members of the Hox family.

**MATERIALS AND METHODS**

**DNA constructs and transgenic analysis**

The HindIII-NotI fragment from a cosmid containing the Hoxa-2 and Hoxa-3 genes was subcloned and mapped. Fragments for transgenic analysis were inserted into the Smal site of the lacZ reporter vector pBGZ40 (Yee and Rigby, 1993; Studer et al., 1994). The respective constructs contained the following genomic fragments: #1, 4.0 kb EcoRI; #2 2.6 kb EcoRI-BglII partial digest; #3 1.9 kb EcoRI-BglII; #4 3.1 kb AccI-EcoRI; #5 1.2 kb BglII-EcoRI; #6 0.8 kb BglII. Construct #7 contained the specific mutant Krox-20 sites. In some cases fragments were also tested using a minimal Hoxb-4/lacZ reporter vector (construct #8 in Whiting et al., 1991). Transgenic mice were generated by microinjection of fertilised eggs from crosses between F1 hybrids (CBA×C57), identified by PCR analysis and embryos assayed for β-galactosidase activity as previously described (Whiting et al., 1991; Sham et al., 1993; Marshall et al., 1994; Studer et al., 1994). For ectopic expression constructs the Krox-20 cDNA was inserted into a 7.5 kb EcoRV fragment containing the Hoxb-1 gene and r4 enhancer (Marshall et al., 1994; Studer et al., 1994; Popperl et al., 1995). The Krox-20 cDNA was inserted into the 5′ untranslated region following removal of sequences from −45 bp upstream to 101 bp downstream of the Hoxb-1 ATG. A line with construct #2 was used to make double transgenic mice in the Krox-20 transactivation experiments. All sequencing was performed by the dideoxy method on both strands.

**Whole-mount in situ hybridisation and combined β-galactosidase staining**

Whole-mount in situ hybridisation was done on mouse embryos as described by Wilkinson and Green (1990); Becker et al. (1994) using a 300 bp Apal-PstI fragment from the 3′ untranslated region of Hoxa-2 cloned into pGemZ to generate a T7 transcribed riboprobe labelled with digoxigenin. β-galactosidase staining on embryos used for in situ hybridisation was performed as previously described (Schneider-Maunoury et al., 1993), except that staining reactions were terminated earlier to prevent masking the in situ signal. The embryo genotype for the Krox-20 mutants was determined by PCR on yolk sac DNA according to Schneider-Maunoury et al. (1993).

**Electrophoretic mobility shift assay (EMSA)**

EMSA and competition assays were performed as described previously with control or Krox-20 containing bacterial extracts (Chavrier
Fig. 1. Time course of Hoxa-2 expression in the developing hindbrain. Dorsal (A,B) and lateral (C) views of 5- (A) and 7-somite (B,C) embryos showing appearance of Hoxa-2 in the presumptive r3 domain. (D) Flat mount of an 8-somite embryo showing the rostrocaudal restriction of the expression in the r3. (E) 10-somite embryo demonstrating activation of Hoxa-2 in r5 and persistence in r3. (F) A dorsal view of a 12-somite embryo showing strong expression in r3 and lower levels in r2, r5 and in the neural crest adjacent to r4. (G,H) Expression in 20- and 25-somite embryos respectively, demonstrating a homogenous low level of Hoxa-2 signal in the neural tube posterior to the r1/r2 boundary, upon which higher levels in r3 and r5 are superimposed. Rhombomeres are indicated by arrows. The number of somites (s) of the embryo is indicated on each panel. nc, neural crest cells; ov, otic vesicle. Scale bar, 200 μm for A-C, G and H, and 100 μm for D and F.

Fig. 2. Effect of the Krox-20 mutation on Hoxa-2 expression in the hindbrain. (A,C-E) Homozygous Krox-20 mutant embryos doubly labelled for Krox-20 by β-galactosidase activity and for Hoxa-2 by in situ hybridisation, demonstrating the absence of Hoxa-2 in r3. The insert in A shows a 5-somite embryo stained for a longer time to show more clearly the presence of Krox-20-expressing cells in r3. (B) Dorsal view of a 5-somite Krox-20⁻/⁻ embryo labelled only with the Hoxa-2 probe showing the absence of transcripts in r3. (F) Lateral view of a 13-somite Krox-20⁻/⁻ embryo labelled only with the Hoxa-2 probe, showing expression in neural crest (nc) cells migrating into the second branchial arch. Note also the presence of Hoxa-2-positive neural crest cells in D-E. (G,H) Double labelling of 12-somite heterozygous (G) and 13-somite homozygous (H) Krox-20 mutant embryos showing colocalisation of Krox-20 and Hoxa-2 in r3 and r5 of the heterozygous embryo and only in r5 of the homozygous mutant embryo, where r3 has already disappeared. Note the presence of Hoxa-2 transcripts in r2, neural crest and posterior neural tube in both embryos. Rhombomeres are indicated by arrows. The number of somites (s) of the embryo is indicated in each panel. nc, neural crest cells. Scale bar, 200 μm.
RESULTS

Hoxa-2 expression in wild-type and Krox-20 mutant embryos

As a first step in determining if Hoxa-2 is subject to regulation by Krox-20 we wanted to examine Hoxa-2 expression in r3 and r5 in Krox-20 mutants. Since the homozygous Krox-20 null mutation leads to a progressive disappearance of r3 and r5 during hindbrain development (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993), the analysis of its effect on gene expression in these rhombomeres is complicated and requires the existence of a window of time during which both the rhombomeres are still present in the mutant and the gene expressed. This is necessary to eliminate the trivial possibility that loss of expression is simply due to the absence of r3 and r5. Therefore, we first had to examine in greater detail the time course of Hoxa-2 expression in these segments in the wildtype hindbrain.

Low levels of Hoxa-2 expression are first observed around 7.5 dp, as previously reported by Frasch et al. (1995). Upregulation of Hoxa-2 in the region of presumptive r3 is detected around the 5-somite stage (Fig. 1A), where its expression continues to increase up to the 15-somite stage (Fig. 1B-F). At the 20- to 25-somite stage expression in r3 begins to decrease (Fig. 1G,H). Upregulation of Hoxa-2 expression in r5 is first detected at the 10-somite stage, and in r5 the levels of expression are generally lower than those in r3. As previously shown, the gene is also expressed at a low level in r2 and r4 from the 12-somite stage and in neural crest emigrating from r4 (Fig. 1 and Hunt et al., 1991; Krumlauf, 1993; Frasch et al., 1995). In homozygous Krox-20 null embryos, we have followed the presence of r3 and r5 cells by the expression of the hybrid Krox-20/lacZ gene resulting from the insertion of the lacZ sequence into the Krox-20 locus (Schneider-Maunoury et al., 1993). The fusion gene expression faithfully recapitulates that of the endogenous Krox-20 gene (Schneider-Maunoury et al., 1993; Topilko et al., 1994). Krox-20/lacZ-expressing cells corresponding to the prospective r3 are observed between the 0- and 10-somite stages (Fig. 2A,C,D and data not shown). In situ hybridisation with a Hoxa-2 probe on a 5-somite stage Krox-20+/- mutant embryo shows that there is no upregulation of Hoxa-2 in prospective r3 even though cells corresponding to future r3 are present (Fig. 2B). This does not represent a delay in upregulation of Hoxa-2 in r3, because we have performed double labelling on embryos at various stages to reveal both β-galactosidase activity and Hoxa-2 mRNA (Fig. 2A,C-H). Presumptive r3 is lost in homozygous Krox-20+/- mutant embryos, as determined by β-gal staining, between the 9- and 11-somite stages (Fig. 2D,E), and no upregulation in r3 was ever detected during this period. Despite these effects on r3, apparently normal Hoxa-2 expression was observed in r2, r4 and r4 neural crest throughout later stages (Fig. 2E-H). Therefore, Hoxa-2 is not upregulated in r3 before it is lost in the Krox-20-/- embryos.

Similar analysis of Hoxa-2 in r5 is more difficult in Krox-20-/- mutants because the level of upregulation in r5 is low in wild-type embryos (Fig. 1E-H) during the period when presumptive r5 cells are still present in the Krox-20+/- mutants (Fig. 2F,H). We have never observed the low level upregulation of Hoxa-2 in r5 in the Krox-20-/- embryos at the 12- to 13-somite stages. Highest upregulation of Hoxa-2 in r5 occurs around the 20- to 25-somite stage in wild-type embryos (Fig. 1H) and we would have liked to examine expression in the mutants at this stage to be sure that upregulation in r5 had not occurred. However in the mutants β-gal staining cells representing presumptive r5 are missing at this stage, precluding analysis (data not shown). In conclusion, our results indicate that Hoxa-2 is under the control of Krox-20 in r3, but with respect to regulation in r5 the data are inconclusive.

Mapping of a Hoxa-2 r3/r5 enhancer

These observed changes in the Hoxa-2 expression pattern raise the possibility that Krox-20 could be directly involved in aspects of Hoxa-2 regulation, in a manner analogous to that previously shown for its paralogous gene Hoxb-2 (Sham et al., 1993). To test this possibility we have used transgenic mice to identify the cis-regulatory regions required for upregulation of Hoxa-2 expression in r3 and r5. Fig. 3 maps the intergenic domain between Hoxa-2 and Hoxa-3 in the mouse where we have identified an r3/r5 control region, and summarises the regulatory regions examined and their activity in transgenic analysis. A 4.0 kb EcoRI fragment upstream of the Hoxa-2 ATG initiation codon was inserted into a vector containing the E. coli β-galactosidase (lacZ) reporter gene under control of a minimal promoter (Construct #1, Fig. 3). The only expression of the transgene in the neural tube was detected in the hindbrain in r3 and r5 (Fig. 4A,B). While there was no expression in r4 itself, the neural crest (nc) cells migrating from r4 into the second branchial arch showed strong staining. Posterior of the otic vesicle and lateral to the neural tube both neural crest and paraxial mesoderm expressed the transgene.

This fragment has been shown to function on several promoters (data not shown), suggesting that there is an r3/r5 enhancer located in the 5′ flanking region and we used deletion analysis to map its position. Constructs #2 and #4 generally had patterns of expression in r3 and r5 and the neural crest identical to those containing the entire 4.0 kb EcoRI fragment (Fig. 4C,E). The 2.7 kb EcoRI-BglII fragment in construct #2 worked in both orientations supporting the idea that the regulatory element has the properties of an enhancer. Construct #4 produced a few embryos (2 of 13) with high levels of staining...
to integration site effects. In construct #7 point mutations (**) in the Krox-20 binding sites have been introduced by site directed mutagenesis.

Restriction sites H, HindIII, R, EcoRI, A, AccI, B, BglII, N, NotI.

in r3 and r5, but only a few positive cells in the r4 neural crest population (Fig. 4F). This suggests that r3/r5 expression is independently regulated from that of the neural crest and has been differentially affected by the integration site in some cases. The only expression from the lacZ reporter in embryos containing constructs #3 and #5 occurred in ectopic locations (Fig. 4D,G). The minimal overlap between constructs #2-5, suggested that the r3/r5 activity of the enhancer might map to an 809 bp BglII fragment, hence we tested its functional activity. Indeed, the BglII fragment alone (construct #6) was sufficient to direct reporter expression in r3 and r5 and the neural crest cells (Fig. 4H).

Analysis of expression mediated by the r3/r5 enhancer

To investigate expression in more detail, four transgenic lines containing the r3/r5 enhancer were generated and displayed identical staining patterns. Fig. 4I-K shows a time course of expression from 8.25-10.5 days post coitum (dpc) for one of the lines. The transgene is initially expressed in r3 at about 8.0 dpc, and a day later high levels of expression are seen in both r3 and r5. Flat mounts and coronal sections show that the expression in the hindbrain is restricted to rhombomeres 3 and 5 (Fig. 5A). Expression then is downregulated in r3 around 10.0 dpc (Figs 4K, 5D), and subsequently in r5 at later stages. The overall temporal progression of appearance and downregulation of expression from the Hoxa-2 transgene mirrors that of Krox-20 (Wilkinson et al., 1989a; Sham et al., 1993), except that it is slightly delayed. This is consistent with the idea that Krox-20 may be directly involved in regulating the Hoxa-2 gene.

Outside the hindbrain, sections reveal that the reporter is expressed in the trigeminal and facial cranial sensory ganglia and mesenchymal cells of the second branchial arch (Fig. 5). In the trigeminal ganglion only a small number of positive cells are observed (Fig. 5E), presumably originating from r3 neural crest, which contributes to a subset of first arch derivatives (Sechrist et al., 1993; Nieto et al., 1995). Sections through posterior regions show that staining occurs in sclerotome and myotome derivatives, lateral mesoderm, and dorsal root ganglia (Fig. 5F). Another site of endogenous Krox-20 expression is the boundary cap cells which surround the exit points of the motor nerves (Wilkinson et al., 1989a; Topilko et al., 1994), and the Hoxa-2/lacZ reporter gene is also expressed in these boundary cap cells (Fig. 5C). Hence the enhancer mediates reporter expression which overlaps with endogenous domains of Krox-20 in a number of locations.

The 809 bp BglII r3/r5 enhancer contains Krox-20 binding sites

On the basis of the boundary cap and r3/r5 restricted expression and similar temporal regulation of the transgene, we investigated the possibility that the BglII fragment might contain Krox-20 binding sites. The fragment was subjected to digestion with HindIII and AvaI, producing four subfragments (Fig. 6A), which were analysed by electrophoretic mobility shift assays (EMSA) using bacterial extract containing the Krox-20 protein. Only the 257 bp HindIII fragment gave rise to retarded complexes (Fig. 6B and data not shown). This suggests that the other fragments do not contain a Krox-20 binding site. Incubation of the 257 bp fragment with increasing concentrations of the Krox-20 protein lead to the formation of two major complexes, C1 and C2 (Fig. 6B). At high concentrations of the extracts, most of the DNA was incorporated into the complex of lowest mobility (C2). Both complexes were competed with an excess of an oligonucleotide carrying a high affinity consensus binding site for Krox-20 (Cons) (Nardelli et al., 1991). No competition was observed with a related oligonucleotide (Mut), having an identical sequence, except for a point mutation which reduces the affinity for Krox-20 (Nardelli et al., 1992) demonstrating the specificity of Krox-20 binding. In conclusion, this analysis suggests that the 809 bp enhancer contains at least two Krox-20 binding sites, both of which are located within the 257 bp HindIII fragment.

Characterisation of the Krox-20 binding sites

Fig. 6A shows the sequence of the 809 bp BglII fragment and the sequence of the 257 bp HindIII fragment in particular was searched for the presence of motifs similar to the high affinity Krox-20 binding site consensus, 5’ GCGNNGGCCG 3’. Two such motifs were identified (Fig. 6C), the first of which (5’ GCGTGGGTG 3’; site #1) is present in reverse orientation. It is very close to the consensus, differing only on the 8th position, which is known to be degenerate because this base is not directly contacted by the protein (Chavrier et al., 1990; Paveletich and Pabo, 1991). The second motif,
The 5’-CTGTGGGCA-3’ (site #2), is more distant from the consensus and was not expected to constitute a high affinity binding site. Both motifs were tested for their capacity to bind Krox-20 in EMSA. Oligonucleotides carrying these motifs were used as competitors against the high affinity Cons oligonucleotide (Fig. 7A). As predicted by the sequence, we found that oligonucleotide 1 carrying the first motif competed almost as efficiently as the Cons oligonucleotide itself and corresponds to a high affinity Krox-20 binding site. In contrast, motif 2 was poorly effective in the competition assay (Fig. 7A). Competitions performed with higher concentrations of oligonucleotide 2 as well as direct binding assays, indicated nevertheless that it bound Krox-20, requiring about ten-fold higher concentrations than the Cons oligonucleotide (data not shown). We also synthesized another oligonucleotide carrying both sites, because sites 1 and 2 are separated by only 20 bp in their natural configuration (Figs 6A, 7B). This combined oligonucleotide was found to be a better competitor that site 1 alone at the same concentrations (Fig. 7A). This suggests that Krox-20 binding to sites 1 and 2 is cooperative and that, in the presence of site 1, site 2 can behave as a high affinity binding site. To establish definitively that the Krox-20 binding activity of the oligonucleotide containing sites 1+2 was only due to the two
Krox-20 directly regulates Hoxa-2

Krox-20 sites identified by the sequence comparison we eliminated them. A related oligonucleotide (1′+2′, Fig. 7B) was synthesized carrying in each of the two sites a single G to C point mutation at the central position of the site, which is known to inactivate binding to the consensus site (Nardelli et al., 1992). EMSA analysis indicated that the mutations had
with consensus high affinity Krox-20 binding site (Cons), was incubated underlined. The labelled probe, an oligonucleotide containing a site is necessary in vivo for the functional activity of the neural crest expression is regulated independently of that in r3 and r5. These results indicate that the two Krox-20 binding sites are required in vivo for r3/r5 enhancer activity, (1 * + 2 * ) to compete for Krox-20 binding (Fig. 7A).

The two Krox-20 binding sites are required in vivo for r3/r5 enhancer activity
In order to determine if the two Krox-20 binding sites defined by the in vitro analysis are involved in the r3/r5 enhancer activity, we introduced the same point mutations tested in vitro (1* + 2*) into the context of the 809 bp BgII fragment (construct #7) for transgenic analysis. Transgene expression in the second arch neural crest and posterior regions was unaffected by the mutations in the Krox-20 sites (Fig. 8). However, in all the embryos generated using the mutated version, staining in r3 and r5 was specifically abolished. Embryos were analysed at a number of stages between 8.0-10.5 dpc to ensure that this was not merely a failure to maintain r3/r5 expression which had been established earlier. Expression in r3 and r5 was never detected at any stage. These patterns of expression mediated by the construct with Krox-20 mutant sites indicate that r4 neural crest expression is regulated independently of that in r3 and r5. These results indicate that the two Krox-20 binding sites are necessary in vivo for the functional activity of the r3/r5 enhancer in the BgII fragment, suggesting that Krox-20 mediates the upregulation of Hoxa-2 in r3 and r5 through interaction with these sites.

Transactivation of Hoxa-2 by Krox-20
Ectopic expression of Krox-20 in transgenic mice was used to examine the ability of Krox-20 to upregulate Hoxa-2 through the r3/r5 enhancer. Using a control region from the Hoxb-1 gene, which directs restricted expression of reporter genes in r4 (Marshall et al., 1994; Studer et al., 1994; Popperl et al., 1995) we generated a transgenic construct (r4/Krox-20) that ectopically expressed Krox-20 in this rhombomeric segment. As shown above (Fig. 4I-K) lines carrying the lacZ reporter under control of the r3/r5 enhancer, do not express the transgene in r4. However when the r4/Krox-20 ectopic expression construct was introduced into this transgenic background, reporter expression was specifically induced in r4 (Fig. 9D,E). Furthermore, this analysis was also performed by co-injecting the r4/Krox-20 construct with the lacZ reporter linked to several versions of the r3/r5 enhancer and identical results were obtained (Fig. 9A-C). In the co-injection experiments we eliminated the possibility that the lacZ reporter activation in r4, was a cis-effect of the r4 enhancer, by using the Hoxb-1 r4 element without Krox-20 as a control (data not shown). In addition we have used a neural enhancer from the Hoxb-4 gene (region A, Whiting et al., 1991) to ectopically express Krox-20 in the neural tube posterior to r6, and found that it also activates the Hoxa-2 r3/r5 enhancer (data not shown). Together our findings demonstrate both that the Krox-20 sites are necessary for enhancer activity and that Krox-20 protein is able to transactivate transgene expression. This argues that the Hoxa-2 gene is a direct in vivo target of Krox-20.

DISCUSSION
The regional identity of segments in the embryonic hindbrain is thought to be regulated by the combinatorial expression of Hox genes, which involves a complex cascade responsible for establishing and maintaining rhombomere-restricted expression. In the present study we have shown that the zinc finger gene Krox-20 is implicated in regulating Hoxa-2 expression in r3 and r5. By deletion analysis in transgenic mice we have defined a 809 bp enhancer with two Krox-20 binding sites shown to be essential for the upregulation of Hoxa-2 in r3 and r5. Furthermore ectopic Krox-20 expression transactivates expression mediated by this enhancer. Thus, our data suggest that Hoxa-2 is a direct target for Krox-20 during the process of hindbrain segmentation.

Krox-20 regulates multiple Hox genes
We found that in Krox-20 mutant embryos there is an absence of Hoxa-2 upregulation in r3, and the loss of expression in r5 is less obvious. In addition, the identification of an r3/r5 enhancer in the 5' flanking region of the gene, which contains Krox-20 binding sites required for its activity, argues that normal upregulation of Hoxa-2 in both r3 and r5 is dependent upon Krox-20. It might be possible that other regulatory elements are involved in regulating r3 and r5 expression of Hoxa-2. However, in our analysis the enhancer described in this paper is the only regulatory region between Hoxa-2 and Hoxa-3 capable of mediating r3/r5 expression. Furthermore, Frasch et al. (1995) scanned a 16 kb genomic region encompassing Hoxa-2 and Hoxa-1 and were unable to find regulatory components involved in mediating the upregulation in r3 and

![Image](image-url)
r5, despite the fact that they reconstructed most of the other domains of Hoxa-2 expression. Therefore, in the Hox complex, analysis over 22 kb spanning the three genes, Hoxa-1 to Hoxa-3, indicates the 809 bp BglII fragment is the only r3/r5 enhancer identified, and we conclude that this regulatory region is likely to be the essential control element responsible for upregulation in r3 and r5 of the endogenous Hoxa-2 gene.

While this data may suggest that Hoxa-2 is important for r3/r5 patterning, the lack of an obvious rhombomeric phenotype in Hoxa-2 null mutants indicates that it is not absolutely required (Gendron-Maguire et al., 1993; Rijli et al., 1993). This does not mean that Hoxa-2 is not involved in regulating r3 and r5 properties, because there could be functional compensation by another Hox gene. In fact our previous analysis on the regulation and expression of its paralog, Hoxb-2, in normal and Krox-20 mutant embryos revealed that in r3 and r5 Hoxb-2 is also directly controlled by Krox-20 (Schneider-Maunoury et al., 1993; Sham et al., 1993). Therefore for normal hindbrain patterning, Krox-20 appears to have a direct role in regulating multiple Hox genes in r3 and r5.

Properties of the Hoxa-2 r3/r5 enhancer

The expression of lacZ reporter genes mediated by the Hoxa-2 and Hoxb-2 enhancers show similar patterns. In addition to the restricted expression in r3 and r5 there are overlaps in a number of domains outside the hindbrain. In particular Hoxa-2 is also expressed in boundary cap cells, which are non-neuronal support cells marking the exit points of the motor nerves. Since Krox-20 mutants also display a phenotype in Schwann cells (Topilko et al., 1994), it is possible that it has a role in regulating Hoxa-2 and Hoxb-2 in locations other than r3 and r5.

Based on our initial analysis the Krox-20 sites of Hoxa-2 and Hoxb-2 alone are not sufficient for r3/r5 regulation. The evolutionary relationship between Hoxa-2 and Hoxb-2 and the fact that they are regulated by Krox-20 would indicate that cooperation with similar or identical factors might be required for the activity of both enhancers. We had hoped to define binding sites for such cooperating factors by using

Fig. 8. Krox-20 binding sites are required for r3/r5 enhancer activity in vivo. (A) Expression in a 9.5 dpc embryo with the wild-type 809 bp r3/r5 enhancer (construct #6). (B,C) Lateral views of reporter expression in two independent transgenic embryos carrying single point mutations in each of the Krox-20 binding sites within the 809 bp enhancer (construct #7). Note that r3 and r5 expression is specifically abolished, and expression in r4 neural crest (nc) and posterior regions is unaltered indicating the Krox-20 sites defined in vitro are necessary for r3/5 enhancer activity only. Below the panels are the sequences of the wild-type and mutant constructs in the region spanning the two Krox-20 binding sites. The arrows indicate the nucleotide changes, which were identical to those used in the EMSA experiments in Fig. 7.

Fig. 9. Ectopic expression of Krox-20 transactivates the r3/r5 enhancer. (A,D) Control embryos from the transgenic line carrying construct #2, showing reporter expression throughout r3 and r5, but not r4. (E) In this transgenic reporter line, the ectopic expression of the Krox-20 protein in r4 specifically induces staining in this rhombomere. (B,C) β-galactosidase expression mediated by various r3/r5 enhancer constructs is also induced specifically in r4 upon ectopic Krox-20 expression in co-injection experiments. nc, neural crest.
sequence comparison to identify conserved blocks that might be required, but this has not been informative. In both the Hoxa-2 and Hoxb-2 enhancers one high affinity Krox-20 binding site has been completely conserved (Fig. 6C, sites #1), but in general there is poor overall conservation of the enhancer nucleotide sequence, including the other Krox-20 binding sites (Fig. 6C). If the sites for common factors are small or moderately degenerate or if different factors are required for Krox-20 interactions in Hoxa-2 and Hoxb-2 regulatory regions, then it may be more important to examine the homologs in other species, which has proved useful for analysis of Hoxb-1 and Hoxb-4 (Marshall et al., 1994; Studer et al., 1994; Aparicio et al., 1995; Morrison et al., 1995; Popperl et al., 1995).

**Regulatory conservation between paralogous Hox genes**

Because Hoxa-2 and Hoxb-2 arose through duplication and divergence from a common ancestor it would not be surprising that they shared common regulatory mechanisms if these arose before the duplication events. However, there are considerable differences in the expression of these two paralogs. Hoxa-2 is strongly expressed in r2 and at low levels in r4, while Hoxb-2 is not expressed in r2 and has high levels in r4 (Krumlauf, 1993). Furthermore, the enhancer mediating r4 expression of Hoxb-2 is located in the 5′ flanking region of the locus and directs expression throughout the rhombomere and its associated neural crest (Sham et al., 1993), while the Hoxa-2 r4 enhancer is positioned within the first intron and mediates only dorsal expression (Frasch et al., 1995). These differences and the lack of conserved sequences in the r4 enhancers, suggests that they reflect independent regulatory mechanisms. Therefore, there has not been a general or global conservation of segmental regulation between these paralogs. Hence, the common role of Krox-20 in regulating r5 expression is a unique highly conserved aspect that presumably reflects a fundamental feature in the regulation of the vertebrate ancestral Hox complex.

**Hoxa-2 regulation in r4 and r4 neural crest**

A primary phenotype of Hoxa-2 null mutants was found to be centred in the neural crest derivatives of the second branchial arch, where there was an anterior homeotic transformation of mesenchymal structures to a first arch identity (Gendron-Maguire et al., 1993; Rijli et al., 1993). This occurred in the absence of any detectable changes to r4, indicating that the neural crest had not adopted an anterior fate due to a change in the identity of the rhombomere from which it was derived. In our analysis, also present in the r3/r5 regulatory region functioned independently of the components in the enhancer required for Krox-20-dependent expression in r3 and r5. Recently, it has been shown that Hoxa-2 has a separate r4 regulatory element in the intron, which directs expression in a dorsal subset of r4 but not in second arch crest (Frasch et al., 1995). This implies that second arch neural crest expression of Hoxa-2 is regulated independently from hindbrain segmentation. In agreement with this, in chick embryos transposed rhombomeres display independent expression in neural tube and neural crest (Prince and Lumsden, 1994). Therefore, the neural crest phenotypes in the Hoxa-2 mutants may reflect a primary role for Hoxa-2 in directing morphogenetic events (mediated by the r4 neural crest enhancer we identified above) in response to signals in the environment of the second branchial arch, rather than a secondary defect from changes in the hindbrain Hox code.

**Are Hox genes the sole or primarily targets of Krox-20 in the hindbrain?**

The rhombomeric phenotypes in Krox-20 mutants could arise solely as a result of the altered regulation of Hoxa-2 and or Hoxb-2 alone. In r3 these are the only members of the Hox family to be expressed. However, members of paralogous group three are also expressed in r5, and in particular Hoxb-3 and Hoxa-3 display a specific upregulation in r5 (Hunt et al., 1991; Keynes and Krumlauf, 1994). Hence, Krox-20 could be involved in regulating even more Hox genes in r5 which contribute to the mutant phenotypes, and it will be important to investigate the mechanism for upregulation of Hoxa-3 and Hoxb-3 in r5.

In addition to Hox genes there are an increasing number of other transcription factors, growth factors and receptor tyrosine kinases which show restricted expression in the hindbrain (Wilkinson et al., 1988, 1989a; Gilardi-Hebenstreit et al., 1992; Becker et al., 1994; reviewed by Wilkinson, 1993). Preliminary evidence in Krox-20 mutants indicates that the Eph family member Sek-1, which is expressed in r3 and r5 (Nieto et al., 1992) is also downstream of Krox-20 in the regulatory cascade (TS and PC, unpublished data). It will therefore be important to determine whether other genes in the hindbrain are controlled directly or indirectly by Krox-20, and in the latter case whether this regulatory link is mediated by Hox genes.

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Krox-20 directly regulates Hoxa-2

553


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